Increased Collagen Synthesis Accompanying Elevated m-RNA Levels in Cultured Werner's Syndrome Fibroblasts

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Although Werner's syndrome (WS) is a premature aging disease and its fibroblasts typically grow poorly in culture, WS may cause abnormalities in connective tissue metabolism that are seldom seen in normal aging, such as scleroderma-like skin. In a preliminary report, we described increased collagen synthesis in fibroblasts derived from two WS patients. The present study was undertaken to determine the degree of the regulation of collagen gene expression in dermal fibroblasts from two other patients. Overproduction of collagenase sensitive protein was observed in WS fibroblasts. Collagen m-RNA levels, that were determined by hybridization of RNA blots with specific cDNA were about 2 times greater than those in the control cells. These results suggest that control of collagen synthesis in WS fibroblasts is altered at the transcriptional level. *J Invest Dermatol* 94:187–190, 1990

Werner's syndrome (WS), an inherited premature aging disease, is assumed to be a disorder of systemic connective tissue metabolism because of many characteristic clinical findings [1,2], such as short stature, calcification of soft tissues, numerous mesenchymal tumors, excessive urinary hyaluronic acid [3,4], and scleroderma-like skin. Fleishmajer [2] noted the existence of homogenized and hyalinized thick collagen bundles in WS skin. Several studies of the connective tissue component using WS fibroblasts have since been carried out [5–9], and we [9] and others [5,8] have shown in preliminary studies that fibroblasts cultured from WS produce increased amounts of collagen, which is the main constituent of the extracellular matrix. Such dermal collagen bundle changes as hyalinization are also seen in keloids and scleroderma. Recent studies have shown that increases in collagen synthesis in fibroblasts derived from some fibrotic disorders, such as scleroderma [10,11] and keloids [12,13], are regulated at the transcriptional level [14,15]. Therefore, it seemed worthwhile to determine whether the collagen gene is activated in WS fibroblasts.

This study was carried out to confirm increased collagen synthesis in WS fibroblasts by testing two WS patients and to examine the collagen gene expression in WS fibroblasts by measuring the cellular steady-state levels of collagen m-RNAs. The results show that fibroblasts cultured from WS produce increased amounts of collagen and that increased collagen synthesis accompanies increased collagen m-RNA, suggesting an alteration in the control of collagen synthesis at the transcriptional level in WS.

MATERIALS AND METHODS

Patients and Skin Fibroblast Cultures Two patients with typical Werner's syndrome (W3: a 43-year-old female; W4: a 57-year-old male) were studied. Their clinical features are presented in Table I. Excision biopsies were performed under local anesthesia from the abdominal skin. Control specimens were obtained from abdominal skin of sex- and age-matched healthy subjects. Primary cultures of the dermal fibroblasts were established by growing the cells on plastic dishes in Dulbecco's modified Eagle's medium (DMEM) containing glutamine (0.6 mg/ml) supplemented with penicillin (100 U/ml), streptomycin (50 μg/ml), and 20% fetal bovine serum (FBS). After outgrowth of cells from the explant culture, subcultures were established by trypsinization (0.2% trypsin, 0.7 mM ethylenediaminetetraacetic acid) of primary cultures and placed in 25 cm^2 plastic flasks. Medium containing 10% FBS was changed every third day, and routine subcultivation was performed at a 1:2 split ratio. All cells were utilized for each study at 8 population doubling level (PDL).

Measurement of Collagen Synthesis PDL-matched WS and normal fibroblasts inoculated onto 35 × 10 mm plastic dishes were assayed at confluency. The culture medium was changed to DMEM supplemented with 50 μg/ml ascorbic acid, 3.4 μg/ml α-ketoglutarate, and 50 μg/ml β-aminopropionitryl containing 5 μCi/ml L-[2,3-^{3}H] proline (Spec. act. 27.7 Ci/mmol, NEN Chemicals) and incubated in a CO_2 incubator for 24 h at 37°C. The number of cells per dish was determined by counting the cells in selected dishes.

Manuscript received April 27, 1989; accepted for publication September 7, 1989.
This work was supported by a grant from the Japanese Ministry of Public Welfare for Scleroderma in 1988.
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Abbreviations:
BSA: bovine serum albumin
CSP: collagenase sensitive protein
10 × Denhardt's solution: 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone
DMEM: Dulbecco's modified Eagle's medium
FBS: fetal bovine serum
GTC: guanidine thiocitrate
Kb: kilobases
NCSP: non-collagenase sensitive protein
PBS: phosphate-buffered saline
PDL: population doubling level
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulfate
5 × SSC: 0.75 M NaCl and 0.075 M sodium citrate
TCA: trichroloacetic acid
WS: Werner's syndrome

0022-202X/90/$03.50

Table I. Clinical Features

Case	Sex	Age	Height (cm)	Weight (kg)	Family history	Cataracta	Diabetes mellitus	Skin sclerosis	Malignancy
W3	F	43	148	35	−	+	−	face extremities	−
W4	M	57	155	40	+	+	+	whole body	−

After labeling, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was added, and the medium was collected. Cell layers were washed with 2 ml of cold phosphate-buffered saline (PBS) and harvested. The medium and the cells were mixed and sonicated. Then collagen synthesis was assayed by measuring the radioactivity of the medium and cells together after limited digestion with purified bacterial collagenase, according to the method of Peterkofsky and Diegelmann [16] with minor modifications. Briefly, 50 μg/ml of bovine serum albumin (BSA) was added as a carrier protein; trichroloacetic acid (TCA) was added to a final concentration of 10%; and the suspension was kept at 0°C for 5 min. After the precipitate was collected by centrifugation at 1000 × g for 5 min, it was resuspended with 1.0 ml of 5% TCA containing 1 mM proline and recentrifuged. The supernatant fraction was then removed, and the procedure was repeated two more times. The precipitates were dissolved and neutralized in 0.05 M NaOH, digested with collagenase form III (Advance Biofactures Corp.) and divided into collagenase sensitive protein (CSP) and non-sensitive protein (NCSP) samples. Then the radioactivity of these samples was counted in a liquid scintillation spectometer. These values, after correction for the number of cells in each dish, were considered as the collagenous protein synthesis and non-sensitive collagenous protein synthesis values.

Determination of m-RNA Levels When the cell layers inoculated onto 150 × 10 mm dishes had achieved confluency, the medium was removed, and the cells were washed two times with cold PBS. To obtain the total cellular extract, 5 M guanidine thiocitrate (GTC) containing 0.75% of 2-mercaptoethanol was then added. Total RNA was isolated by centrifugation over a cushion of 5.7 M cesium chloride [17]. Constant amounts of RNA (1 μg/lane) were separated by electrophoresis on 1% agarose/formamide gels transferred to a nitrocellulose filter for Northern hybridization following standard procedures [18]. For slot blot analyses RNAs (5–0.3125 μg) were denatured in buffer containing formaldehyde for 15 min at 65°C and applied to nitrocellulose filters using a vacuum slot-blot template. Filters were then baked for 2 h at 80°C under a vacuum and prehybridized for 3 h at 42°C in 10 × Denhardt's solution containing 50% formamide, 5 × SSC, 0.05 M phosphate (pH 6.5), and sonicated salmon sperm DNA (250 μg/ml). Specific hybridization was carried out for 24 h at 42°C, as described elsewhere [19], using ^{32}P-labeled cDNA probes specific for the α1(I), α1(III) collagen chains and β-actin. The filters were washed twice in 2 × SSC, 0.1% SDS at room temperature, twice in 0.1 × SSC, 0.1% SDS at 62°C, air dried, and exposed to x-ray films at −80°C for various periods of time. The uptake of radioactivity was determined by scanning autoradiograms with a densitometer. Plasmids Hf-677 with a 1.5-Kb insert specific for the α1(I) collagen chain [20], plasmids pIII-33 with a 0.9-Kb insert for the α1(III) collagen chain [21], and plasmids pHFA-1 with a 0.5-Kb insert for β-actin [22] were used as DNA probes for collagen I, III and β-actin m-RNA.

Statistical Analysis Statistical significance of the data was calculated using the Student t test.

RESULTS

Collagenous and Non-Collagenous Protein Synthesis of WS Fibroblasts The results are presented in Table II. We demonstrated collagen synthesis and non-collagen synthesis by adding up the values per 10^4 cells, after which we calculated the collagen synthesis/total protein synthesis ratio. The collagen synthesis in the WS cells (W3, W4) was approximately 1.5-fold greater than that in the control cells (N3, N4). The collagen synthesis/total protein synthesis ratio in the WS cells was 1.2 times greater than that in the control cells.

Collagen m-RNA Level of WS Fibroblasts The results of Northern blotting with W4 and N4 are shown in Fig 1. Each specific band of α1(I) and α1(III) collagen in W4 was shown to be greater than those in N4 despite the same level of β-actin. A more quantitative comparison, which is summarized in Table III, was then made with the slot blot assay (Fig 2). The m-RNA levels of W3 and W4 showed a 2.04-fold (average) increase in α1(I) collagen and a 1.67-fold (average) increase in α1(III) collagen over control values. The m-RNA levels of β-actin, on the other hand, showed a tendency to decrease.

DISCUSSION

Because Werner's syndrome may cause abnormalities in connective tissue metabolism [2], several investigations of WS cell cultures in vitro have been carried out. We described increased collagen synthesis in fibroblasts derived from two WS patients in a preliminary report [9], and two other preliminary reports [5,8] observed its increase in fibroblasts from WS patients. In this study, we also showed increased collagen production in fibroblasts from two other patients. The other main component of the extracellular connective tissue matrix is glycosaminoglycan (GAG), and increased total GAG synthesis [6,9] and accumulation [6] on the cell surface of the fibroblasts of WS patients has also been reported. Bauer et al [23] noted that the constitutive levels of collagenase in WS fibroblasts were sixfold greater than in control fibroblasts. It is a well-known

Table II. Collagen Synthesis by Dermal Fibroblasts from Patients with Werner's Syndrome[a]

Cell	CSP(dpm/10^4 cells)	NCSP(dpm/10^4 cells)	CSP/CSP + NCSP
W3	2258 ± 109	2102 ± 195	0.52
N3	1355 ± 87	1854 ± 146	0.42
	($p < 0.01$)		
W4	2494 ± 95	1543 ± 82	0.61
N4	1610 ± 90	1357 ± 110	0.53
	($p < 0.01$)	(W3, N3 n = 4 W4, N4 n = 5)	

[a] Fibroblast cultures from WS patients (W3, W4) and age, sex, and PDL-matched normal controls (N3, N4) were labeled with [^{3}H] proline for 24 h. After labeling the cells with [^{3}H]-proline, medium and cell proteins were precipitated with trichroloacetic acid, digested with purified bacterial collagenase (Form III Advance Biofactures Corporation), and divided into collagenase sensitive protein (CSP) and non-sensitive protein (NCSP). Radioactivities were then determined as described in *Materials and Methods*, and CSP/CSP + NCSP ratios were calculated. These values were corrected for the number of cells in each dish. Each value represents the mean ± SEM.

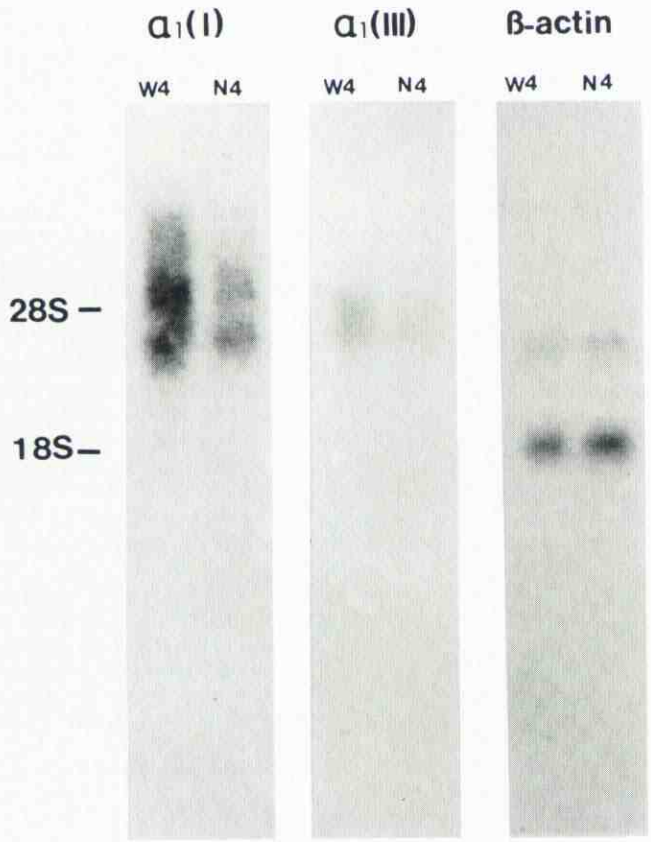

Figure 1. Northern blot detection of α1(I), α1(III) collagen, and β-actin specific m-RNAs obtained from a WS patient (W4) and age-, sex-, and PDL-matched normal control (N4) dermal fibroblasts. Total RNAs (1 μg each) were electrophoresed and transferred to nitrocellulose, and m-RNAs were hybridized to labeled cDNA. The positions of 28s and 18s RNA are indicated by *bars*.

fact that normal human diploid fibroblasts show a loss of the ability to synthesize collagen [24,25] and total GAG [26] and to induce collagenolytic activity [24] after continued in vitro aging. These facts therefore seem to indicate that the connective tissue metabolism in fibroblasts from WS is different from that in cultured aging fibroblasts.

Another aging syndrome, Hutchinson-Gilford progeria, is also associated with scleroderma-like skin, and recently it was reported [27] that tropoelastin production by progeria fibroblasts was elevated at the protein and m-RNA levels, while relative collagen synthesis was similar to control fibroblasts. Progeria fibroblasts have been found to have an abnormal connective tissue metabolism, but there are differences in the abnormal metabolism of these two premature aging disorders.

Table III. m-RNA Levels Specific for Collagen I, III, and β-actin of Fibroblasts[a]

	Collagen α1 (I) (%)	Collagen α1 (III) (%)	β-actin (%)
W3	207	175	83
N3	100	100	100
W4	201	158	82
N4	100	100	100

[a] Slot blot filters were exposed to x-ray film and the intensity of hybridization was quantitated densitometrically. Values were expressed as percent compared to normal controls. All values were normalized for the same amount of RNA.

Abnormalities of connective tissue metabolism are known to occur in scleroderma and/or keloids. Fibroblasts cultured from scleroderma, a disease of the connective tissue leading to fibrosis of the skin and other involved organs, exhibit an increase in collagen synthesis [10,11]. Recently elevated cellular levels of the collagen m-RNAs [28] and an increased transcription rate [14] of the corresponding gene in scleroderma fibroblasts have been reported. Fibroblasts cultured from keloids, which are dermal fibrotic lesions, also exhibit an increase in collagen synthesis [12,13] associated with elevated cellular levels of the corresponding m-RNAs [15]. In this study, increased levels of type I collagen m-RNA were also found in WS fibroblasts producing increased amounts of type I collagen. It remains to be seen whether an increase in the transcription rate or increased stability of these m-RNAs is responsible for the higher m-RNA levels. The increases in the amount of type I and III m-RNA were 100% and 70%, respectively. The difference between the m-RNA levels of type I and type III suggests that production of type I collagen is largely controlled by the level of the corresponding m-RNA. A similar situation has been reported by Uitto et al [15] in keloid fibroblasts. CSP production in WS fibroblasts was about 60% higher than in control fibroblasts. There was little difference between increases in collagen synthesis and elevations of corresponding m-RNA levels. Therefore, it is suggested that the gene expression of collagen is altered mainly at the transcriptional level and a translational control may also be present.

The authors thank Dr. A. Matsumiya for helpful discussions, Y. Yoshida for technical assistance, and K. Oda for typing the manuscript.

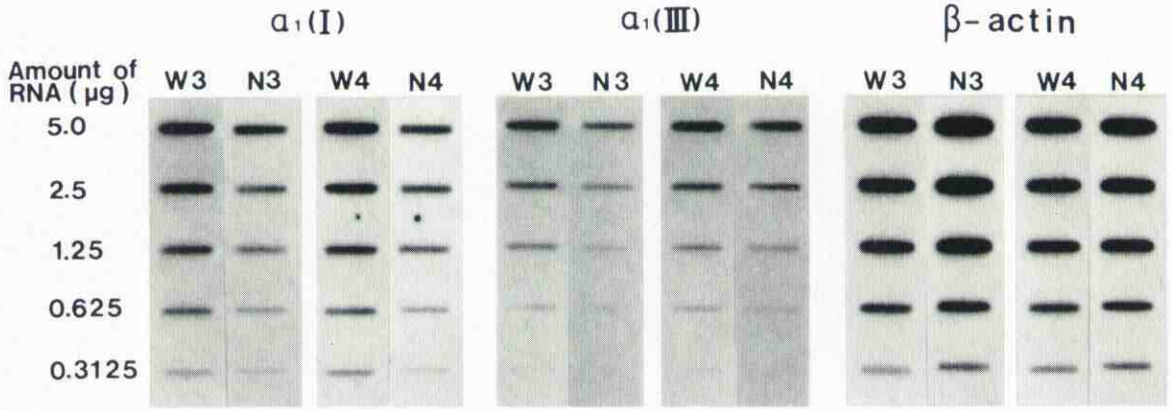

Figure 2. Blot quantification of m-RNAs of dermal fibroblasts derived from WS patients (W3, W4) and age-, sex-, and PDL-matched normal controls (N3, N4). Serial dilutions of total RNA (5, 2.5, 1.25, 0.625, 0.3125 μg) were dotted onto a nitrocellulose filter, baked, and hybridized with cDNA probes of α1(I), α1(III) collagen, and β-actin m-RNA.

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